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1-β-D-Arabinofuranosylcytosine Inhibits Borna Disease Virus Replication and Spread

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Borna disease virus (BDV) is a nonsegmented, negative-strand RNA virus that causes neurological diseases in a variety of warm-blooded animal species. There is general consensus that BDV can also infect humans, being a possible zoonosis. Although the clinical consequences of human BDV infection are still controversial, experimental BDV infection is a well-described model for human neuropsychiatric diseases. To date, there is no effective treatment against BDV. In this paper, we demonstrate that the nucleoside analog 1-β-D-arabinofuranosylcytosine (Ara-C), a known inhibitor of DNA polymerases, inhibits BDV replication. Ara-C treatment inhibited BDV RNA and protein synthesis and prevented BDV cell-to-cell spread in vitro. Replication of other negative-strand RNA viruses such as influenza virus or measles virus was not inhibited by Ara-C, underscoring the particularity of the replication machinery of BDV. Strikingly, Ara-C treatment induced nuclear retention of viral ribonucleoparticles. These findings could not be attributed to known effects of Ara-C on the host cell, suggesting that Ara-C directly inhibits the BDV polymerase. Finally, we show that Ara-C inhibits BDV replication in vivo in the brain of infected rats, preventing persistent infection of the central nervous system as well as the development of clinical disease. These findings open the way to the development of effective antiviral therapy against BDV.

Borna disease virus (BDV) is a nonsegmented, negative-strand (NNS) RNA virus (14, 43) that has the property, unique among animal *Mononegavirales*, of transcribing and replicating its genome in the nucleus (12). BDV is highly neuronotropic but may also replicate in other cells of the central nervous system (CNS) (6, 30). In vitro, cells of different types and species are susceptible to BDV infection. BDV is noncytolytic in all cell systems examined so far (24).

BDV is the causative agent of Borna disease, a neurological disorder of horses, sheep, and other farm animals (30). Recent evidence indicates that the natural host range as well as geographic distribution and prevalence of BDV are much broader than previously thought (41). The spectrum of clinical diseases due to BDV infection ranges from subtle behavioral abnormalities (e.g., impairment of learning and memory) to progressive, immune-mediated meningoencephalitis (21). There is considerable evidence that BDV infects humans (4, 15, 33, 42), and infection has been claimed to be associated with certain neuropsychiatric disorders (3, 4, 28, 42). However, the epidemiology and the clinical consequences of human infection remain controversial (5).

To date, there is no effective treatment against BDV. Amantadine was initially reported to have some antiviral activity (2). However, this result could not be confirmed in other studies (11, 22, 45). Recently, two studies reported the inhibitory effect of the broad-spectrum antiviral ribonucleoside analogue ribavirin on BDV replication (25, 31). Treatment with ribavirin decreased the production of cell-free BDV as well as the levels of viral RNA transcripts in persistently infected cell lines. However, the efficacy of ribavirin was limited and was tested only on persistently infected cell lines and not in vivo.

1-β-D-Arabinofuranosylcytosine (Ara-C) is a nucleoside analogue that differs from cytosine by the presence of a hydroxyl group at the 2′ position of the sugar residue. Its active metabolite, Ara-CTP, inhibits cellular and viral DNA polymerases (18) and can be incorporated into DNA (9). Ara-C is commonly used in the treatment of leukemias. Although Ara-C is active against a variety of DNA viruses (8), activity against an RNA virus has never been reported.

In this study, we demonstrate that Ara-C potently inhibits the production of cell-free BDV and abolishes BDV spread in persistently infected cells, as well as in newly infected primary hippocampal neurons. Inhibition of viral spread was associated with decreased levels of viral RNA and protein synthesis. Immunofluorescence and biochemical analyses demonstrate that Ara-C treatment induced a dramatic change in the subcellular distribution of viral proteins, consistent with the retention of viral ribonucleoparticles (RNP) in the nucleus. Ara-C-induced retention of viral RNP could not be mimicked by leptomycin B (LMB), an inhibitor of the CRM-1-mediated nuclear export pathway. Moreover, the antiviral effect of Ara-C could not be attributed to any of its known effects on the host cell. Therefore, Ara-C most likely acts directly on the BDV polymerase. As expected, Ara-C was not active against other negative-strand RNA viruses such as influenza virus and measles virus. The specific sensitivity of BDV to the DNA polymerase inhib-

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itor Ara-C emphasizes the particularity of its replication machinery. Finally, we show that Ara-C inhibits BDV replication in vivo in the brain of infected rats, thereby preventing the development of clinical disease.

MATERIALS AND METHODS

Cells and viruses. Vero, C6, U373, and Madin Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1 (Gibco) and supplemented with 10% fetal calf serum (FCS) and 1× Bufferall (Sigma), with the exception of MDCK cells, which were grown in DMEM supplemented with 5% FCS. Primary hippocampal neurons were isolated from 1-day-old Sprague-Dawley rats (Janvier). Briefly, hippocampi were dissected and dissociated by mechanical trituration and digestion with phosphate-buffered saline (PBS) plus 0.3% aspergillus protease (Sigma) for 10 min at room temperature. DNase (Sigma) was then added to a final concentration of 1 mg/ml and incubated for 10 min at room temperature. After addition of 35% FCS, the suspension was passed over a 70-μm cell strainer and centrifuged at 400 × *g* for 10 min. After resuspension in Neurobasal medium (Gibco), the cell suspension was centrifuged at 400 × *g* through a 4% bovine serum albumin cushion for 10 min. Thereafter, cells were seeded on poly-D L-ornithine (Sigma), laminin (Roche)-coated glass coverslips and grown in Neurobasal medium supplemented with 25 mM glutamate, 25 mM β-mercaptoethanol, 0.5 mM glutamine, 2% B-27 supplement (Gibco), and 2% FCS. After 24 h in culture, the mitotic inhibitors 5-fluoro-2′-deoxyuridine (Sigma; 10 mg/ml) and uridine (Sigma; 25 mg/ml) were added to limit growth of glial cell contaminants. BDV infection was performed by adding cell-free virus in the culture medium. We used BDV laboratory strain He/80, measles virus strain Edmonston B (kindly provided by F. Tangy), and influenza virus type A strains Puerto Rico 8/34 (H1N1) and Sydney 5/97(H3N2), kindly provided by N. Naffakh.

Reagents and antibodies. 1-β-D-Arabinofuranosylcytosine, ribavirin, and LMB were purchased from Sigma. Aphidicolin and camptothecin were purchased from ICN. As primary antibodies we used anti-microtubule-associated protein 2 (MAP-2) mouse monoclonal (Sigma), anti-nucleoprotein (N) and anti-phosphoprotein (P) rabbit polyclonals (23), and anti-N mouse monoclonal (38/17C1) (46) antibodies. Fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin G (IgG), Cy3-labeled anti-mouse IgG (Interchim), peroxidase-labeled anti-rabbit IgG (Amersham), and biotinylated goat anti-rabbit IgG as well as *R*-phycoerythrin-labeled streptavidin were purchased from Southern Biotechnology Associates.

Preparation of cell-free BDV and titration. Cells were treated with 10 mM HEPES (pH 7.2 to 7.5) with 250 mM $MgCl_2$ for 90 min at 37°C to enhance viral release (19). Supernatants were collected, and the virus was purified by ultracentrifugation (36,000 × *g* for 135 min) through a 20% sucrose cushion in 10 mM HEPES, 75 mM NaCl, 2 mM EDTA (pH 7.2). Titration of cell-free BDV (reported in focus-forming units [FFU] per milliliter) was performed as described before (19).

Cell-to-cell spread assay for BDV. Vero cells were grown to confluence, incubated for 45 min with 5 μM 5-(and-6)-carboxyfluorescein diacetate (CFDA; Molecular Probes), and washed two times for 30 min with medium without CFDA. CFDA is a fluorescent label that is retained in living cells through several generations and that is not transferred among adjacent cells in a population. CFDA passes freely across the cell membrane, but once inside the cell it undergoes rapid enzymatic modification producing a cell-impermeant reaction product. Persistently infected unlabeled Vero cells (Vero-BV) were then added at a ratio of 1:1 and cocultivation was carried out for 5 days. Thereafter, cells were dissociated with trypsin, harvested, and fixed in 2% paraformaldehyde for 15 min at room temperature. After fixation, cells were incubated twice for 10 min in reaction buffer (PBS–1% bovine serum albumin–0.5% saponin) at room temperature and blocked for 45 min in 7% normal goat serum in reaction buffer at room temperature. Cells were incubated for 1 h with anti-N polyclonal antibody in reaction buffer on ice, washed extensively in reaction buffer, incubated for 30 min with biotinylated goat anti-rabbit IgG on ice, washed extensively, and incubated with *R*-phycoerythrin-labeled streptavidin in reaction buffer on ice. After extensive washing in reaction buffer and PBS, cells were analyzed using a FACScalibur.

Immunofluorescence. Cells grown on glass coverslips were fixed for 30 min at room temperature with 4% paraformaldehyde followed by 10 min at room temperature with methanol-acetone (1:1). Thereafter, cells were rinsed with PBS and blocked overnight at 4°C with PBS plus 2% normal goat serum and 2% normal horse serum. Incubation for 1 h at room temperature with primary antibodies was followed by a 1-h incubation at room temperature with the secondary antibodies. After extensive washes, sections were mounted using Vectashield (Vector Laboratories).

Northern blotting. RNA was isolated using TriReagent following the standard protocol and analyzed by Northern blotting as previously described (23). Briefly, 5 to 10 μg of RNA per slot was separated under denaturing conditions on a 1% agarose gel (containing 2.2 M formaldehyde), and RNA was transferred onto a positively charged nylon membrane (Micron Separations, Inc.). Thereafter, blots were hybridized with 10^7 precipitable cpm of ^{32}P-labeled, random-primed probes, washed at high stringency (0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.2% sodium dodecyl sulfate [SDS] at 65°C) and exposed. For quantitation, blots were analyzed using a PhosphorImager (Molecular Dynamics).

SDS-PAGE and immunoblotting. Cells were lysed in protein lysis buffer containing 10 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $ZnCl_2$, 100 mM Na_3VO_4, 1 mM dithiothreitol, and a tablet of protease inhibitor cocktail (Complete, Mini, EDTA-free; Roche). Protein samples were standardized for protein content using a Bradford assay. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE; 10% bis-Tris), transferred onto Hybond membranes by electroblotting, and immunoblotted with the appropriate antibodies. Revelation was done using a Pierce Supersignal Chemoluminescent kit as described previously (23).

Subcellular fractionation. Subcellular fractionation was done as previously described (36). Briefly, cells were taken up in IsoHi buffer (Tris-buffered saline–10 mM $MgCl_2$–protease inhibitors) plus 0.5% NP-40 (vol/vol) and incubated for 2 min on ice under gentle shaking. Cells were centrifuged at 1,000 × *g* for 5 min at 4°C and the supernatant was transferred to a clean tube. The pellet was washed in IsoHi buffer containing 0.66% Tween 20 and 0.33% sodium deoxycholate for 2 min on ice under gentle shaking. After centrifugation at 1,000 × *g* for 5 min at 4°C the supernatant was pooled with the previous supernatant (cytoplasmic fraction). The nuclear pellet was taken up in protein lysis buffer as described above.

In vivo studies. Eight-week-old Lewis rats were infected intracranially with 1,000 FFU of BDV strain He/80 and were treated either with PBS (mock) or with Ara-C. The treatment schedule was as follows: preloading at day −1 (one day prior to infection) with 200 mg of Ara-C/kg of body weight intraperitoneally (i.p.), thereafter gradually decreasing maintenance doses at days 0, 1, 3, and 5 with 50 mg of Ara-C/kg i.p.; at days 7, 9, 11, 13, and 15 with 25 mg of Ara-C/kg i.p.; and at days 17, 19, 21, and 23 with 10 mg of Ara-C/kg i.p. Animals were examined daily for the development of clinical symptoms. Clinical scores were defined as follows: 0, no signs; 0.5, ruffled fur and very mild hunchback; 1, slight incoordination and fearfulness; 2, distinct ataxia or slight paresis; 3, paresis or a significant degree of paralysis; 4, death. At day 25 postinfection, brain tissue was collected for determination of viral titers, analysis of BDV RNA levels, and histological analysis.

RESULTS

Ara-C specifically inhibits BDV replication and spread in vitro. In order to analyze the potential effect of Ara-C on BDV replication, we determined cell-free viral titers in the medium of a monkey kidney cell line (Vero) persistently infected with BDV (Vero-BV) after daily treatment with different concentrations of Ara-C or of 20 μM ribavirin (25). Since BDV spreads predominantly by cell-to-cell contact, very little cell-free virus is present in cell culture. To determine the production of cell-free virus, BDV-infected cells were briefly treated by osmotic shock before collection of the supernatant (19). The toxicity threshold for Ara-C, which was defined as the concentration causing ≥5% cell death (as assessed by trypan blue staining), was ≈10 μM Ara-C/day. When cells were treated with 1 μM Ara-C or more, viral titers declined rapidly (Fig. 1, top panel). In cells treated with 2 or 4 μM Ara-C, viral titers were reduced by more than 1,000-fold. Treatment with 20 μM ribavirin reduced titers of cell-free BDV only by ≈25%. We observed a similar effect of Ara-C on the production of cell-free BDV in other cell lines persistently infected with BDV, such as rat (C6) and human (U373) astroglioma cells (data not shown).

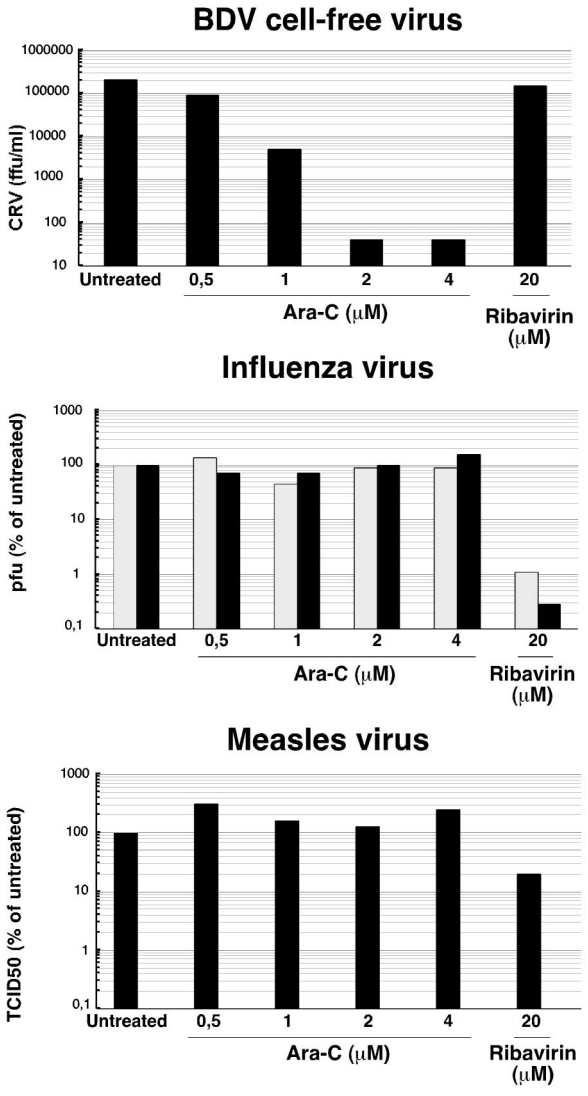

FIG. 1. The effects of Ara-C and ribavirin on the titers of cell-free BDV, influenza virus, and measles virus. (Top) BDV cell-free viral titers, in FFU per milliliter of isolate. (Middle) Influenza virus strain Sydney (▨) and strain Puerto Rico (■) viral titers in PFU are given as the percentages of untreated controls. (Bottom) Measles virus strain Edmonston B viral titers (50% tissue culture infective dose; Kärber method) are given as the percentages of untreated controls.

To test whether the effect of Ara-C was specific for BDV or applied also to other negative-strand RNA viruses, we analyzed the effect of Ara-C on the replication of influenza virus and measles virus in cultured cells. The cytopathic nature of both viruses, even when we infected at a low multiplicity of infection (MOI), prevented treatment for more than 3 days before collecting the medium for viral titration. MDCK cells were infected with influenza virus strain Sydney or Puerto Rico (MOI, 0.001) and treated daily with different doses of Ara-C or with 20 μM ribavirin. Influenza virus production was not inhibited by Ara-C, whereas ribavirin reduced the viral production of both strains by at least 2 log units (Fig. 1, middle panel). We tested the effect of Ara-C on measles virus by treating Vero cells infected with measles virus strain Edmonston B (MOI, 0.05) with different doses of Ara-C or with 20 μM ribavirin. Ara-C treatment did not reduce measles virus production, whereas ribavirin reduced viral production by up to 80% (Fig. 1, bottom panel).

Since BDV infectivity is tightly cell associated (20), efficient transmission of BDV is likely to require cell-to-cell contact and may be due primarily to bare RNP rather than to enveloped virions (12). Therefore, we developed a new assay to analyze the effect of potential viral inhibitors on cell-to-cell spread of BDV. We labeled confluent layers of Vero cells with CFDA and subsequently cocultivated them for 5 days with unlabeled Vero-BV cells. Cocultivation took place under daily treatment with different doses of Ara-C or with 20 μM ribavirin. The percentage of the total cell population that was positive for both CFDA as well as for BDV-N staining was taken as an index for viral spread. Whereas ribavirin treatment reduced viral spread only moderately (≈25%), treatment with 2 to 4 μM Ara-C almost completely abolished BDV spread (Fig. 2).

Ara-C inhibits viral RNA and protein synthesis and causes viral RNP retention in the nucleus. We next assessed the effects of Ara-C treatment on viral RNA levels by analyzing total cellular RNA by Northern blotting using cDNA probes for BDV-N and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a host cell housekeeping gene (Fig. 3, top panel). GAPDH, BDV mRNA, and genomic BDV RNA levels were quantified with a PhosphorImager and standardized to GAPDH levels (Fig. 3, middle panel). We observed a progressive inhibition of both genomic RNA (up to 77% inhibition) and viral mRNA (up to 80% inhibition for the 1.2-kb transcript coding for BDV-N) upon treatment with Ara-C. In addition, Western blotting experiments showed that the expression of BDV-N and BDV-P were drastically decreased upon Ara-C treatment (Fig. 3, bottom panel).

The effect of Ara-C on the subcellular distribution of viral proteins was striking. We immunostained Ara-C- and ribavirin-treated Vero-BV cells with anti-N (Fig. 4) and anti-P (data not shown) polyclonal antibodies. In untreated Vero-BV cells, both proteins were localized in the cytoplasm as well as in the nucleus. With increasing concentrations of Ara-C, both N and P proteins progressively disappeared from the cytoplasm and accumulated in the nucleus. Ribavirin treatment caused comparable, but less drastic, effects on the subcellular distribution of BDV proteins (Fig. 4). We confirmed these results by subcellular fractionation of Vero-BV cells, followed by Western blot analysis for N and P proteins (Fig. 4, bottom panel). The steady decrease in the total amount of viral proteins in Ara-C-treated cells was accompanied by a dramatic change in the subcellular distribution of viral proteins. Since both N and P are basic constituents of viral RNP, their nuclear localization is indicative of nuclear retention of viral RNP. Withdrawal of Ara-C treatment for 1 to 2 days resulted in a partial recovery of the original subcellular distribution of BDV proteins, indicating that the effect might be—at least in part—reversible (data not shown).

Studies using cell lines transfected with BDV-N and/or BDV-P have shown that BDV-N is a nucleocytoplasmic shuttling protein that may play a pivotal role in the nuclear export of RNP (27). Since BDV-N makes use of the CRM-1-mediated pathway for nuclear export, an interference of Ara-C with this pathway might explain the observed change in subcellular dis-

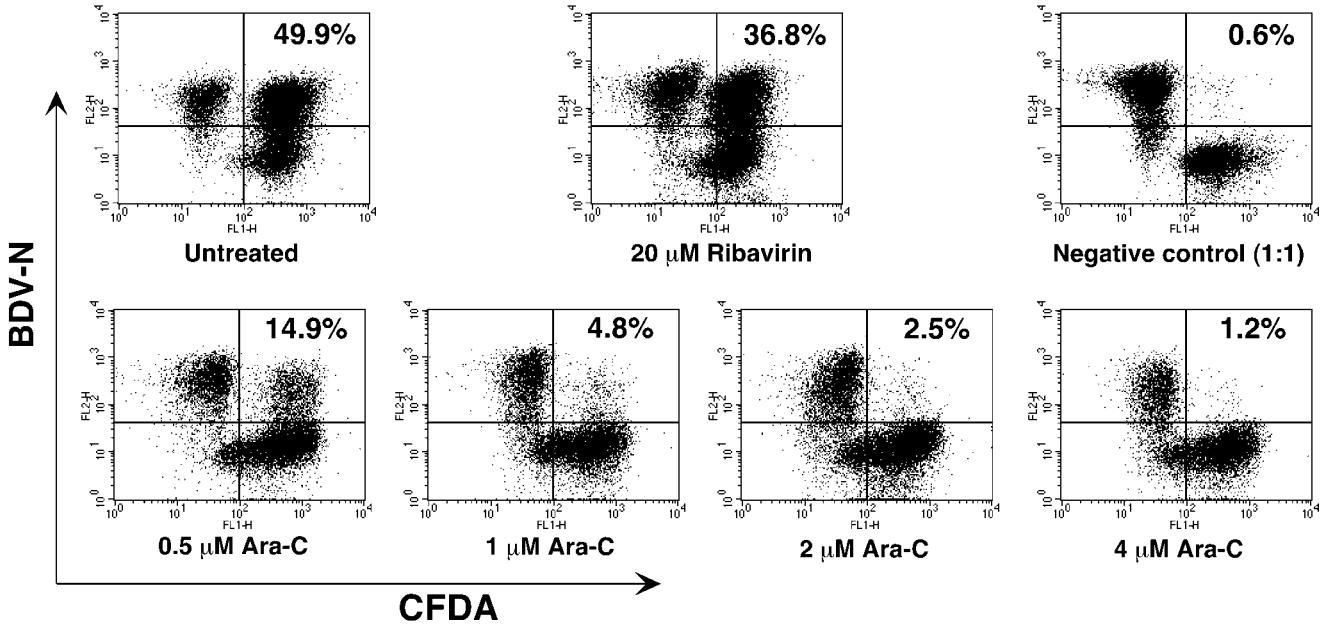

FIG. 2. Ara-C inhibits BDV cell-to-cell spread. Confluent Vero cells were labeled with CFDA and subsequently cocultivated for 5 days at a ratio of 1:1 with unlabeled, BDV-infected Vero cells (Vero-BV). Cocultivation took place under daily treatment with different doses of Ara-C or with 20 μM ribavirin, after which cells were analyzed by flow cytometry. The percentages shown indicate the double-positive population within the population of viable cells, which indicates the amount of viral spread. The negative control consisted of a 1:1 mix of CFDA-labeled Vero cells with Vero-BV cells, fixed directly after mixing.

tribution of BDV antigens. Therefore, we examined if Ara-C affected the subcellular distribution of IκB-α, a protein known to be exported out of the nucleus by CRM-1 (40). The subcellular distribution of IκB-α was not affected by treatment with up to 4 μM Ara-C for up to 5 days, whereas the known CRM-1 inhibitor LMB caused complete nuclear redistribution of IκB-α within 3 h (Fig. 5). In addition, treatment with LMB did not alter the intracellular distribution of BDV antigens in Vero-BV cells (Fig. 5), not even when cells were treated for up to 48 h with concentrations of up to 200 nM LMB (data not shown).

Ara-C inhibits BDV dissemination ex vivo in primary hippocampal neurons. Since, in vivo, BDV replicates and persists predominantly in neurons of the limbic system (21), we isolated primary hippocampal rat neurons, infected them ex vivo with cell-free BDV, and analyzed the effects of Ara-C on BDV replication and spread. Since primary hippocampal neurons were more sensitive to the cytotoxic effects of repetitive Ara-C treatment than were established cell lines, we changed the treatment schedule from daily to a single dose of 1 or 2 μM Ara-C, given at day 1 postinfection. Ribavirin treatment was done on a daily basis since no cytotoxicity was observed. Use of a low MOI (approximately 0.025 FFU/cell) allowed us to test for viral spread in the culture. We fixed cell cultures at days 4, 5, 6 (data not shown), and 7 (Fig. 6, top panel) postinfection and stained them for both viral proteins and the neuronal cell marker MAP-2. Untreated cultures contained 25% infected neurons at day 7, whereas ribavirin- and Ara-C-treated cultures contained, respectively, 7.5% and 0.1% infected neurons. In addition, the change in subcellular distribution of N protein seen in Vero-BV cells was also observed in Ara-C-treated neurons (Fig. 6, bottom panels).

The antiviral effect of Ara-C cannot be attributed to its effects on the host cell. The inhibitory effects of Ara-C on BDV replication and dissemination might be caused by direct effects on the viral replication machinery or by interference with host cell functions that the virus needs for its replication or spread. The antimitotic activity of Ara-C is the basis for its use in the treatment of leukemias. Two mechanisms have been described to contribute to its toxic effects on dividing cells. First, the active metabolite of Ara-C, Ara-CTP, is a competitive inhibitor of DNA polymerase-α and -β (18, 47). Second, incorporation of Ara-C into DNA traps topoisomerase I-induced cleavage complexes, thereby inhibiting topoisomerase I-mediated DNA religation (39).

To investigate whether the inhibitory effects of Ara-C could be attributed to its antimitotic effects on the host cell, CFDA-labeled Vero cells were cocultivated with unlabeled Vero-BV cells and treated with the general mitotic inhibitors 5-fluoro-2′-deoxyuridine and uridine. BDV-spread was assessed by fluorescence-activated cell sorter analysis. Despite the apparent inhibition of cell division, treatment with mitotic inhibitors did not inhibit BDV spread (Fig. 7). The inhibitory effect of Ara-C on DNA polymerase-α and -β can be mimicked by treatment with aphidicolin, whereas its inhibitory effect on topoisomerase I-mediated DNA religation can be mimicked by treatment with camptothecin (38). Therefore, Vero-BV cells were treated with either of these reagents at the highest possible noncytotoxic dose, which was defined as ≤5% cell death (as assessed by trypan blue staining). Neither aphidicolin nor camptothecin treatment resulted in inhibition of viral spread (Fig. 7).

If the inhibitory effect of Ara-C were caused by a direct effect on the viral replication machinery, the BDV-L would be

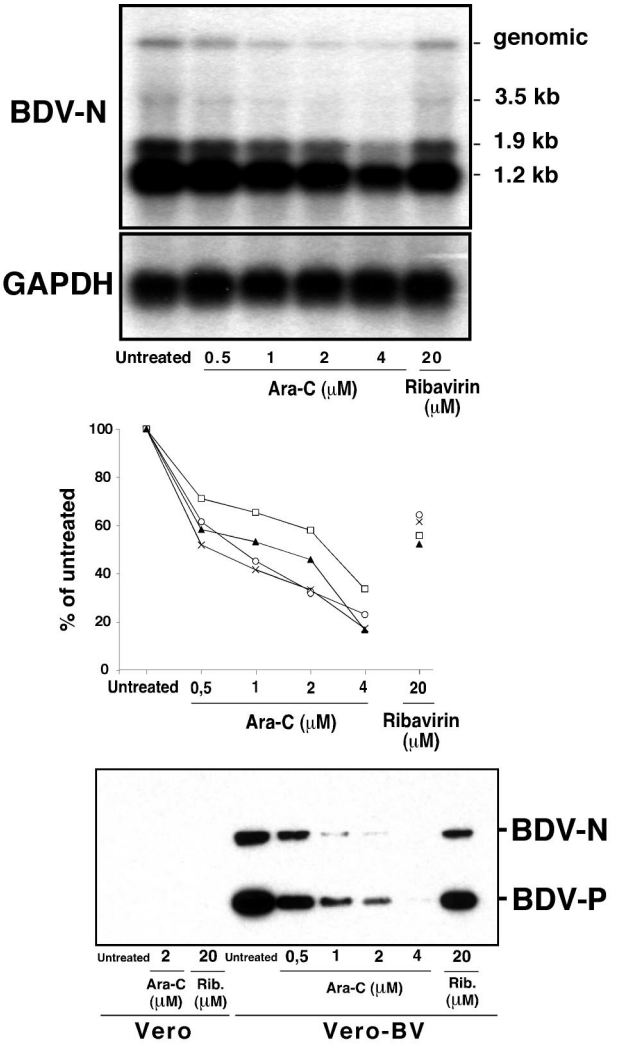

FIG. 3. BDV RNA and protein expression levels are inhibited by Ara-C. Total cellular RNA and proteins from BDV-infected Vero cells were extracted at day 5 of treatment and analyzed. (Top) Northern blot analysis. The expected sizes of the different BDV mRNAs and genomic RNA are indicated. (Middle) Quantitation of BDV mRNA and genomic BDV RNA levels was done by PhosphorImager analysis and was thereafter standardized to GAPDH levels. Results are expressed as the percentages of untreated control levels, which were set to 100%. ○, genomic RNA; □, mRNA of 3.5 kb; ▲, mRNA of 1.9 kb; and ×, mRNA of 1.2 kb. (Bottom) Western blot analysis. Equal amounts of protein were resolved by SDS-PAGE, transferred to a Hybond membrane, and reacted with anti-N and anti-P antibodies (as indicated).

the most likely target. Ara-C could directly inhibit the activity of the L polymerase. Alternatively, Ara-C could exert a mutagenic effect during RNA synthesis mediated by BDV-L, resulting in the generation of high levels of nonfunctional viral genomes. Such a mechanism has recently been described to underlie the inhibitory activity of ribavirin on poliovirus replication (10). Therefore, we tested whether Ara-C treatment of Vero-BV cells would increase the rate of mutations in the BDV genome. By using reverse transcription-PCR, we amplified the 3′ end of genomic viral RNA derived from Ara-C-treated and nontreated Vero-BV cells. Thereafter, we determined the sequences of 450-bp stretches of eight viral clones derived from Ara-C-treated cells and of seven viral clones derived from nontreated cells, and we analyzed the results (data not shown). We could find no evidence for increased mutation frequencies in genomic BDV RNA sequences derived from Ara-C-treated cells (0.17%) compared to that in nontreated sequences (0.15%).

Ara-C inhibits BDV replication in vivo and prevents persistent CNS infection and clinical disease. Encouraged by the robust inhibitory effects of Ara-C on BDV replication and dissemination in primary hippocampal neurons, we decided to study its effects in vivo. Four groups of five Lewis rats were studied. Ten animals were infected intracranially with 1,000 FFU of BDV (strain He/80) and five of them received Ara-C i.p., whereas the other five were mock treated (PBS; i.p.). The other 10 animals were mock infected (PBS); five of them received Ara-C treatment and the other five were mock treated. The treatment schedule was designed to give minimal Ara-C-related side effects. We examined rats daily for the development of clinical symptoms up to 25 days postinfection. At that time, we sacrificed the rats and collected brain tissue for determination of viral titers, analysis of BDV RNA levels, and histological analysis. All five rats treated with PBS and infected with BDV developed clinical symptoms characteristic of Borna disease (Table 1). By contrast, all Ara-C-treated rats only showed a clinical score of one until the end of the experiment. These mild symptoms were also seen in Ara-C-treated, mock-infected rats and were most probably due to the well-documented side effects of Ara-C. Viral titers from mock-treated,

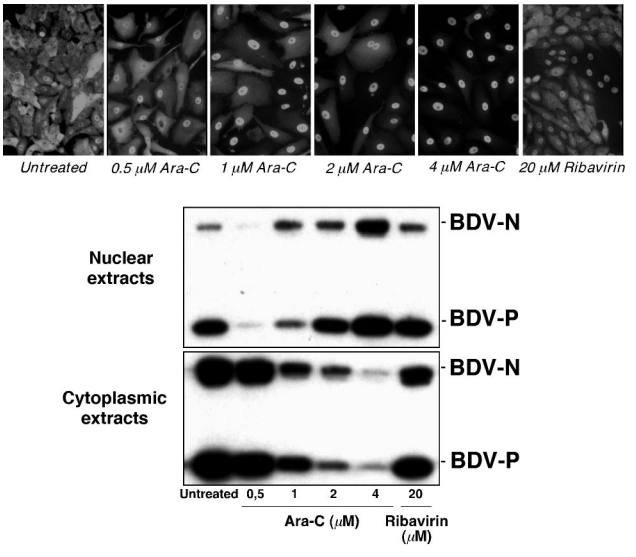

FIG. 4. The subcellular distribution of BDV viral proteins is dramatically changed by Ara-C treatment. (Top) Vero-BV cells at day 5 of treatment were stained with anti-N antibody followed by FITC-conjugated anti-rabbit antibody. Original magnification, ×200. Similar results were obtained with C6 and U373 cells as well as with the 38/17C1 monoclonal antibody against N or a polyclonal antibody against BDV-P (data not shown). (Bottom) Cell fractionation followed by Western blot analysis. Vero-BV cells at day 5 of treatment were lysed and lysates were separated into nuclear and cytoplasmic fractions. Equivalent amounts of protein were subjected to SDS-PAGE, transferred to a Hybond membrane, and reacted with anti-N and anti-P antibodies (as indicated).

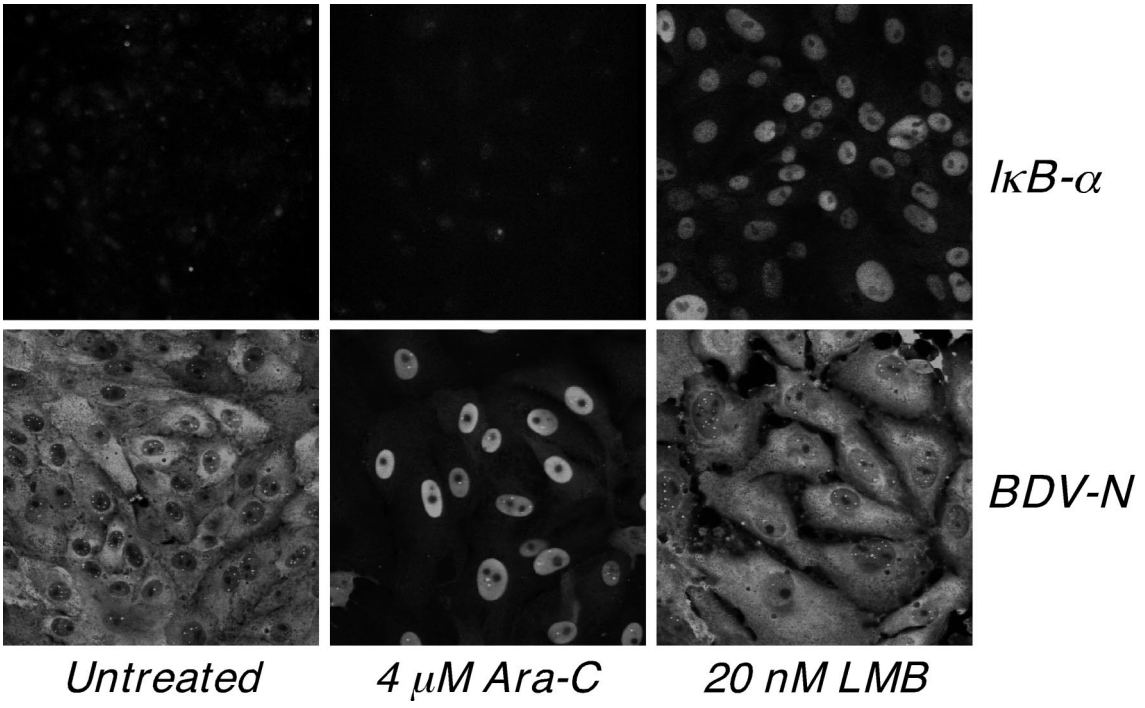

FIG. 5. Analysis of the effect of Ara-C and LMB on the subcellular localization of IκB-α and BDV-N. Vero-BV cells were left untreated, treated with 20 nM LMB for 3 h, or treated with 4 μM Ara-C for 5 days. Cells were fixed and analyzed by immunofluorescence for the expression of IκB-α or BDV-N. Similar results were obtained with staining for the BDV-P (results not shown).

BDV-infected rats were 5.6 (± 1.5) × 10^5 FFU/g of brain tissue, whereas viral titers from Ara-C-treated, BDV-infected rats were below the detection level. Likewise, we could easily detect by Northern blotting BDV RNA in the brains of all (5 of 5) mock-treated BDV-infected animals, whereas no viral RNA was detectable in BDV-infected rats treated with Ara-C (Fig. 8, top panel). Finally, histological analysis of brain tissue from mock-treated BDV-infected rats showed characteristic inflammation associated with BDV infection (21), whereas no inflammation could be detected in Ara-C-treated, BDV-infected rats (Fig. 8, bottom panels).

DISCUSSION

BDV is the causative agent of Borna disease, a neurological disorder that affects horses, sheep, and other farm animals (21). Experimentally, BDV can cause CNS diseases, mainly manifested by behavioral abnormalities, in a large number of vertebrate species. The best-studied model systems are infection of the adult rat, in which BDV causes classical immune-mediated CNS disease (34, 44), and infection of the newborn rat, in which persistent infection of limbic structures causes subtle behavioral disturbances (1, 7, 16, 34). BDV's broad host range, the isolation of BDV from human peripheral blood mononuclear cells, and accumulating data on human seropositivity for BDV make it a candidate for zoonosis with, as yet, unknown clinical consequences (4, 15, 17, 26, 29, 33, 37). This, in turn, provides the impetus to search for drugs with activity against BDV.

In this paper, we describe a drug with a strong activity against BDV both in vitro and in vivo. In vitro, Ara-C inhibited the production of cell-free BDV by more than 1,000-fold and eliminated cell-to-cell spread. Both viral RNA and viral protein levels were decreased and there was a dramatic change in the subcellular distribution of viral proteins, consistent with nuclear retention of viral RNP. In primary cultures of hippocampal neurons, Ara-C inhibited BDV cell-to-cell spread considerably and sequestered viral RNP in the nucleus of infected neurons. In vivo, Ara-C prevented viral replication in the brain of infected rats and the development of clinical disease. Replication of other negative-strand RNA viruses such as influenza virus or measles virus was not inhibited by Ara-C, underscoring the particularity of the replication machinery of BDV. Based on this study, treatment might be developed that could potentially eradicate persistent BDV infection from the CNS.

Since Ara-C is a nucleoside analogue known to inhibit cellular and viral DNA polymerases, we thought it unlikely at first that it would directly interfere with the replication of an RNA virus. This, combined with the fact that Ara-C did not inhibit replication of two other negative-strand RNA viruses (influenza virus and measles virus; Fig. 1), made us test the hypothesis that the antiviral effect of Ara-C toward BDV could be mediated by its effect on the host cell rather than by an effect on viral replication.

Ara-C is a powerful antimitotic drug (8). To test whether this affected BDV replication, we induced total growth arrest of Vero-BV cells using conventional mitotic inhibitors (5-fluoro-2′-deoxyuridine and uridine). This did not inhibit BDV cell-to-cell spread (Fig. 7). Also, Ara-C exerted a potent anti-BDV effect on nondividing, primary hippocampal neurons

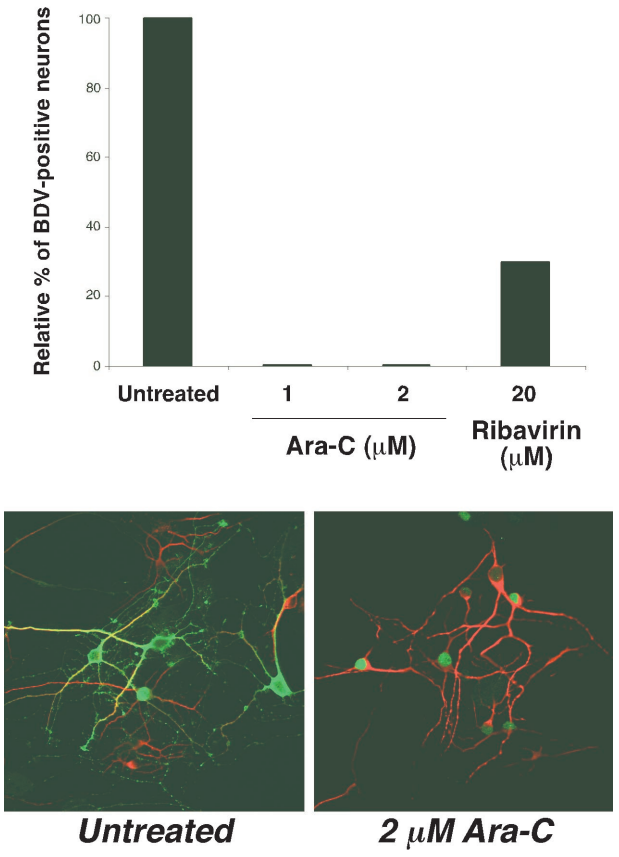

FIG. 6. The effect of Ara-C and ribavirin on BDV dissemination and on the subcellular localization of BDV-N in primary hippocampal neurons. The graph on top shows results for primary rat hippocampal neurons at day 7 postinfection (MOI, 0.002) that were doubly stained for BDV-N and the neuronal cell marker MAP-2. Percentages of MAP-2-positive neurons that were also positive for BDV-N were determined. Results are given as percentages of untreated controls. Immunofluorescence images show primary rat hippocampal neurons at day 7 postinfection (MOI, 0.025) doubly stained for BDV-N (green) and MAP-2 (red). Original magnification, ×200.

(Fig. 6). Therefore, our data are in agreement with earlier reports that demonstrate that cell growth arrest by serum starvation has no inhibitory effect on BDV, but on the contrary might even enhance viral replication (32). We also mimicked the inhibitory effects of Ara-C on the DNA polymerase-α and-β of the host cell (18, 47) and on the religating properties of topoisomerase I (39) by treating Vero-BV cells with aphidicolin, an inhibitor of DNA polymerase-α and -β or camptothecin. Neither viral spread (Fig. 7) nor the subcellular localization of N and P proteins was affected (data not shown) by either treatment.

The change in subcellular localization of BDV proteins caused by Ara-C, both in persistently infected cells and in acutely infected neurons (Fig. 4 and 6), indicates nuclear retention of RNP. This retention could explain the reduced yields of cell-free BDV (Fig. 1) as well as the strong inhibition of BDV dissemination (Fig. 2 and 6), and it might account for a considerable part for the antiviral effect of Ara-C. Since BDV-N uses the CRM-1-mediated pathway for nuclear export, interference of Ara-C with this pathway would have been the simplest explanation for the observed nuclear retention of viral RNP. However, we found no evidence for Ara-C-mediated inhibition of the CRM-1 pathway for nuclear export (Fig. 5). Moreover, Ara-C had no effect on influenza virus replication, which is sensitive to inhibitors of CRM-1-mediated nuclear export (35). In addition, treatment with LMB did not alter the intracellular distribution of BDV antigens in Vero-BV cells (Fig. 5). This discrepancy with previously reported LMB-mediated inhibition of BDV-N nuclear export could be due to the fact that our studies were performed with infectious virus, whereas Kobayashi et al. used cell lines transfected with individual BDV proteins (27). Taken together, it is unlikely that Ara-C exerts its anti-BDV effect by interfering with the host cell machinery. However, we cannot formally exclude the possibility that other unknown cellular pathways, important for BDV replication, might be affected by Ara-C.

We favor the hypothesis that Ara-C has a direct effect on BDV-L. Although little is known about L polymerase, it is only distantly related to other *Mononegavirales* polymerases (13). Therefore, it is possible that its mode of replication and transcription, and by consequence its sensitivity to drugs, is different from that of other nonsegmented, negative-stranded viruses. We found no evidence that Ara-C has a mutagenic effect during RNA synthesis mediated by the BDV polymerase, resulting in the accumulation of nonfunctional viral genomes. Therefore, the most likely explanation for the antiviral effect of Ara-C on BDV replication and spread is that Ara-C directly inhibits BDV-L. Direct inhibition of L activity by Ara-C could explain the comparable decrease in viral mRNA and genomic levels seen upon Ara-C treatment (Fig. 3), since both transcription and replication processes are dependent on BDV-L. Inhibition of BDV-L by Ara-C and the subsequent decrease in viral genomic RNA could also explain the nuclear retention of BDV-N and -P. It has been proposed that, during viral replication, N shuttles between the nucleus and the cytoplasm (27), mediating the nuclear export of RNP complexes. The formation of viral RNP complexes depends on the availability of both

TABLE 1. Clinical symptoms in mock-treated and Ara-C-treated BDV-infected rats

Day postinfection	Ara-C	BDV	Clinical evaluation score[a] (n[b])
	−	−	0 (5)
5	−	+	0 (5)
	+	−	1 (5)
	+	+	1 (5)
	−	−	0 (5)
15	−	+	1 (5)
	+	−	1 (5)
	+	+	1 (5)
	−	−	0 (5)
20	−	+	1 (1); 2 (4)[b]
	+	−	1 (5)
	+	+	1 (5)
	−	−	0 (5)
25	−	+	2 (3); 3 (2)
	+	−	1 (5)
	+	+	1 (5)

[a] Clinical scores were defined as: 0, no signs; 0.5, ruffled fur and very mild hunchback; 1, slight incoordination and fearfulness; 2, distinct ataxia or slight paresis; 3, paresis or a significant degree of paralysis; 4, death.
[b] Number of animals with that given score.

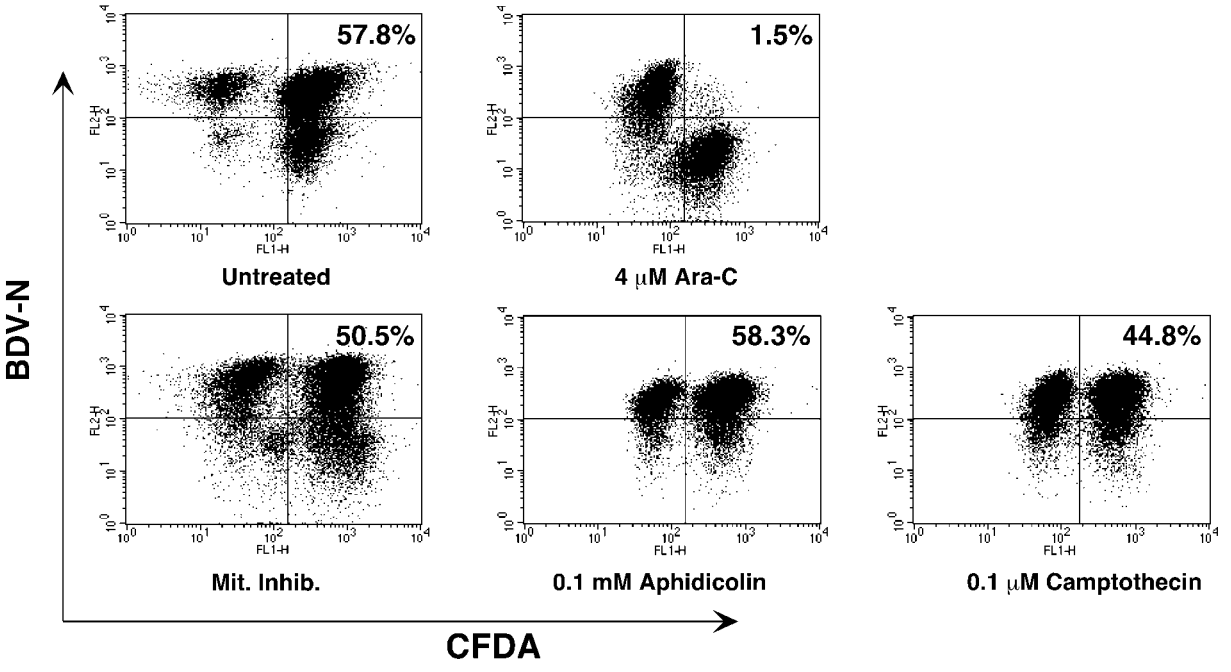

FIG. 7. Flow cytometric analysis of the effect of treatment with mitotic inhibitors aphidicolin or camptothecin on BDV cell-to-cell spread. Confluent Vero cells were labeled with CFDA and subsequently cocultivated for 5 days with unlabeled, BDV-infected Vero cells (Vero-BV). Cocultivation took place under daily treatment with 4 μM Ara-C, 0.1 mM aphidicolin, or 0.1 μM camptothecin or under treatment every other day with mitotic inhibitors (10 mg of 5-fluoro-2′-deoxyuridine/ml and 25 mg of uridine/ml). The percentages shown indicate the double-positive population within the population of viable cells, which indicates the amount of viral spread. The negative control consisted of a 1:1 mix of CFDA-labeled Vero cells with Vero-BV cells, fixed directly after mixing.

viral proteins and viral genomic RNA. In the absence of viral genomic RNA in the nucleus, BDV-N and -P will complex by the binding of P to the nuclear export signal of N, thereby causing nuclear retention of both proteins (27).

Recently, two reports have described the inhibitory effects of ribavirin on BDV replication in vitro (25, 31). Ribavirin was the only nucleoside analogue with anti-BDV activity out of a variety of analogues tested. However, its efficacy against BDV was moderate and was tested only on cell lines. Here, side-by-side comparison of ribavirin with Ara-C shows that Ara-C is more efficient in all systems tested. Of the two anti-BDV agents reported previously, the effect of amantadine is controversial and the effect of ribavirin is moderate. Moreover, neither of them has been reported to be effective in vivo and, as for all other known persistent viral infections of the CNS, there is no effective treatment at present for BDV. In this study, we demonstrate that Ara-C successfully inhibited BDV replication in the brain of acutely infected rats (Table 1; Fig. 8). More importantly, Ara-C also inhibited the development of clinical symptoms (Table 1) as well as the development of the characteristic inflammatory response (Fig. 8).

Our studies show, unexpectedly, that the DNA polymerase inhibitor Ara-C is a potent inhibitor of the negative-strand RNA virus BDV. New, Ara-C-based nucleoside analogues, with similar anti-BDV efficacy and less toxicity, might be considered as attractive candidates for the development of anti-BDV therapy. However, given the controversial issue of human BDV infections, it is clear that further studies are needed to support Ara-C treatment of humans with suspected BDV infections.

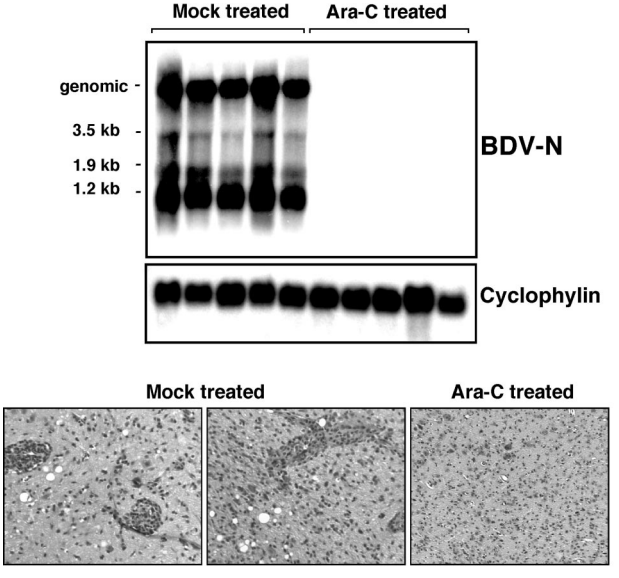

FIG. 8. Ara-C inhibits BDV replication in the brains of rats infected with BDV and prevents the development of persistent CNS infection. (Top) Northern blot analysis. Rats were infected intracranially with BDV and treated with either Ara-C or PBS (mock). At 25 days postinfection rats were sacrificed and total cellular RNA was extracted from brain tissue. Northern blots were probed against cyclophylin (as a housekeeping gene) and BDV-N. The expected sizes of the different BDV mRNAs and genomic RNA are indicated. (Bottom) Histology of brain samples collected from rats infected with BDV and treated with either Ara-C or PBS (mock). Ten-micrometer-thick sections were stained with hematoxylin and eosin and analyzed for the presence of inflammatory infiltrates characteristic for viral persistence in the CNS. Original magnification, ×100.

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